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Lou, Yan-Ru

2018

Lou , Y-R & Leung , A W 2018 , ' Next generation organoids for biomedical research and applications ' , Biotechnology Advances , vol. 36 , no. 1 , pp. 132-149 . https://doi.org/10.1016/j.biotechadv.2017.10.0

http://hdl.handle.net/10138/327408
https://doi.org/10.1016/j.biotechadv.2017.10.005

Title

Next Generation Organoids for Biomedical Research and Applications

Authors

Yan-Ru Lou[1,*], Alan W. Leung[2,*]

Affiliation

[1] Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland

[2] Yale Stem Cell Center and Department of Genetics, Yale School of Medicine, Yale University, New Haven, Connecticut, United States

*** Correspondence**

Alan W. Leung, Yale Stem Cell Center and Department of Genetics, Yale School of Medicine, Yale University, New Haven, Connecticut, United States

Email: alan.leung@yale.edu

Yan-Ru Lou, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland

Email: yan-ru.lou@helsinki.fi

Abstract

Organoids are *in vitro* cultures of miniature fetal or adult organ-like structures. Their potentials for use in tissue and organ replacement, disease modeling, toxicology studies, and drug discovery are tremendous. Currently, major challenges facing human organoid technology include (i) improving the range of cellular heterogeneity for a particular organoid system, (ii) mimicking the native micro- and matrix-environment encountered by cells within organoids, and (iii) developing robust protocols for the *in vitro* maturation of organoids that remain mostly fetal-like in cultures. To tackle these challenges, we advocate the principle of reverse engineering that replicates the inner workings of *in vivo* systems with the goal of achieving functionality and maturation of the resulting organoid structures with the input of minimal intrinsic (cellular) and environmental (matrix and niche) constituents. Here, we present an overview of organoid technology development in several systems that employ cell materials derived from fetal and adult tissues and pluripotent stem cell cultures. We focus on key studies that exploit the self-organizing property of embryonic progenitors and the role of designer matrices and cell-free scaffolds in assisting organoid formation. We further explore the relationship between adult stem cells, niche factors, and other current developments that aim to enhance robust organoid maturation. From these works, we propose a standardized pipeline for the development of future protocols that would help generate more physiologically relevant human organoids for various biomedical applications.

Keywords

Organoids; Human; Reverse Engineering; Self-organization; Designer Matrix; Niche Factors; Pluripotent Stem Cells; Drug Screening; Disease Modeling; Transplantation

1. INTRODUCTION

Strong predictability of preclinical testing is vital to success in clinical trials. Current preclinical tests for efficacy, toxicity, and pharmacokinetics are far from perfect. While animal models have been regarded as the gold standard, the use of laboratory animals continues to pose ethical questions. The considerable animal species differences in reactions to drugs (Burkina et al., 2017) and in disease phenotypes (Seok et al., 2013) also significantly lower the accuracy of preclinical predictions. For some diseases—such as infectious diseases—no relevant animal models are available. It has been reported that only 8% of the predictions of cancer drugs generated from animal models have been successfully translated into human clinical trials (Mak et al., 2014). In addition to animal models, *in vitro* two-dimensional (2D) cell cultures are also widely used for preclinical testing. The predictions from 2D cell cultures, however, are often difficult to interpret and could be misleading because cells cultured in 2D environments often lose their functionality and have altered phenotypes that are different from those in *in vivo* tissues and organs. In recent years, the United States Environmental Protection Agency (EPA), the National Institute of Health (NIH), and the Defense Advanced Research Projects Agency (DARPA) have initiated programs such as the ToxCast Programme and the Microphysiological Systems (MPS) Program to advocate the development of human cellular models to assess the safety and/or the efficacy of environmental chemicals (Kleinstreuer et al., 2014) and drugs (Fabre et al., 2014). In particular, the DARPA MPS program promotes the development of organs-on-chips and human-on-chips approaches to synthesize *in vitro* three-dimensional (3D) human tissues derived from cultured cells on bioengineered platforms to bring together native-like tissue architecture and physiology for highly-predictive and physiologically-relevant monitoring of the functions and effects of toxic substances and drugs at the organ- or body-levels. There is an urgent need to develop more physiologically relevant, efficient, and robust protocols to advance technology that synthesizes human tissues.

1.1 DEFINING ORGANOIDS. The term *organoid* was first employed in an oncology study to indicate a pathological and tumor-like tissue mass formed in a human infant (Kretzschmar and Clevers, 2016; Smith and Cochrane, 1946). The term has since been used in the medical field to describe a multisystem disorder, called *organoid nevus syndrome*, related to benign outgrowths of sebaceous glands on the skin and the eye of affected individuals (Shields et al., 1996). *Organoid* was loosely used in experiments to denote transplanted tissues or the resultant tissue masses from transplants (Waddell, 1949; Yoshida et al., 1980). Increasingly frequent in the late 1980s and early 1990s, *organoid* was employed in developmental biology experiments to denote high-density or organ-like cultures generated through aggregation and cell sorting of dissociated animal cells and tissues (Elkasaby et al., 1991; Ridgeway et al., 1986; Schroter-Kermani et al., 1991). The two decades following this were characterized by the advent of embryonic (ESC) and induced pluripotent (iPSC) stem cell cultures derived from rodent, primate, and human embryos (Martin, 1980; Thomson et al., 1998; Thomson et al., 1995) and somatic cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007; Yu et al., 2007), as well as the discovery of adult stem cells in various tissues including retina (Tropepe et al., 2000), bone marrow (Wilson and Trumpp, 2006), epidermis (Clayton et al., 2007), intestinal crypts (Clevers, 2013), stomach glands (Leushacke et al., 2013), testis (Klein et al., 2010), esophagus (Doupe et al., 2012), and brain (Fuentealba et al., 2012). Following these successive breakthroughs, the definition of *organoid* evolved to describe the *in vitro* 3D structures derived from differentiating cultures of ESCs, iPSCs, or adult stem cells that bear an *in vivo* tissue-like arrangement, compartmentalization, and functionality. In this review, we adopt

this definition and will use the term *organoids* to describe the *in vitro* cultures of conglomerates of tissue-specific cells that incorporate cell-cell and cell-matrix interactions in an orderly and 3D manner. In most cases described here, *organoids* were cultured either in suspension or embedded in animal-derived matrices such as Matrigel. *Organoids* can be generated from clonal derivatives of adult stem cells or aggregates of stem cells and organ-specific progenitors derived from primary embryonic cell types, ESCs, or iPSCs. In regular cultures, depending on the types of organs, *organoids* can be spherical or irregularly shaped masses ranging in diameter from 0.1 to 1 mm. They demonstrate significant homology in terms of tissue architecture and gene expression profiles compared to their *in vivo* counterparts. They also display growth, undergo morphogenesis, mature, and in many cases, exhibit functionality similar to their tissues of origin and have the ability to integrate into embryonic and adult tissues upon transplantation.

1.2 MAJOR TRENDS AND CHALLENGES IN CURRENT ORGANOID RESEARCH. Studies involving organoid generation have rapidly evolved in recent years, branching into a field going into multiple directions. There are studies using organoids to understand the pathology of a number of genetic disorders including cystic fibrosis (Dekkers et al., 2013; Hohwieler et al., 2017), polycystic kidney disease (Freedman et al., 2015), Hirschsprung's disease (Workman et al., 2017), and neurodevelopmental defects like microcephaly and lissencephaly (Bershteyn et al., 2017; Lancaster et al., 2013). Organoid-driven approaches have been employed to investigate disease mechanisms of infectious pathogens like *Helicobacter* (McCracken et al., 2014) and Zika virus (Cugola et al., 2016; Dang et al., 2016; Garcez et al., 2016; Li et al., 2017; Qian et al., 2016). Not surprisingly, organoids derived from intestinal and hepatic cell types have been examined as a possible source of *in vitro* tissues for regenerative medicine, and these studies have been met with great success in numerous animal models (Fordham et al., 2013; Fukuda et al., 2014; Huch et al., 2015; Takebe et al., 2014; Yui et al., 2012). More recently, toxicology screens (Schwartz et al., 2015; Takasato et al., 2015) and studies on patient-specific genetic factors and drug responses in tumorigenesis have begun employing organoid technology (Cristobal et al., 2017; Fujii et al., 2016; Li et al., 2014; Matano et al., 2015; van de Wetering et al., 2015; Verissimo et al., 2016; Weeber et al., 2015).

ESCs, iPSCs, and, to a certain extent, adult stem cells provide a virtually unlimited source of raw cellular materials for organoid generation. In the case of ESCs and iPSCs, organoids are derived either via the embryoid body method, in which pluripotent stem cell colonies are lifted up from undifferentiated adherent cultures and fragmented into small pieces for further differentiation (Freedman et al., 2015; Kadoshima et al., 2013; Lancaster and Knoblich, 2014; Lancaster et al., 2013; Muguruma et al., 2015; Nakano et al., 2012; Suga et al., 2011) or directly from sub-structures formed on differentiating adherent cultures (Calderon-Gierszal and Prins, 2015; Dye et al., 2015; McCracken et al., 2014). Despite these advances in technique, there are still tissues that are recalcitrant to organoid derivation such as the epidermis, the testes, the ovaries, the bladder, the thyroid gland, and other non-epithelial tissues such as the heart and the skeletal muscles (Fatehullah et al., 2016). Additional challenges demand improvements to current organoid technology. Organoid derivation often results from the tedious and unguided testing of empirical conditions for proper differentiation, yielding a long and costly generation process. Heterogeneity in viability, size, and shape of the derived organoids plagues the development of high throughput systems and algorithms that can be employed for phenotypic, toxicologic, and drug screens. In many cases, techniques used for generating one type of organoid cannot be easily transferred to a different organoid system, e.g. the procedures in developing a brain organoid will bear little resemblance to those for a liver organoid. This lack

of transferability of organoid techniques across systems hinders the development of the organoid field and limits its potential to incorporate other fields of study such as computational science and bioengineering that require the building of mathematical/engineering models and the incorporation of scalable and cross-system parameters. Importantly, organoids usually lack the co-induction of the essential cell types and the associated extracellular matrices and native microenvironment that will allow the recapitulation of the *in vivo* tissue sizes, structures, organization, inter-cellular communication, and functionality. Lastly, very little is known about the culturing conditions and specific factors driving the *in vitro* maturation of organoids, which in many cases remain fetal in nature.

1.3 APPLYING THE CONCEPT OF REVERSE ENGINEERING TO ORGANOID GENERATION. In biology, reverse engineering is the process of extracting the minimal set of design parameters from existing biological systems for creating a functional mimicry (Ingber, 2016; Shinozawa et al., 2016). Design parameters may include cell type, soluble microenvironment, insoluble microenvironment, and physical parameters such as shape, external force, and fluid flow. We propose that by employing the principle of reverse engineering, we can solve some of these issues, in particular: (i) recapitulating the *in vivo* cellular heterogeneity in organoid systems, (ii) controlling the organoid microenvironments including extracellular matrix (ECM) composition, stiffness, and architecture, and (iii) promoting the *in vitro* maturation of organoids. Here, we review current developments and techniques concerning the generation of human organoids and propose that by studying and employing the minimal elements required for the different stages of *in vitro* organoid synthesis and maturation, we can improve and streamline current organoid generation approaches as depicted in Figure 1.

2 EXPLOITING THE SELF-ORGANIZING PROPERTY OF EMBRYONIC PROGENITORS

Self-organization has been widely demonstrated in animal studies, whereby tissue-specific progenitors, after random dispersal, aggregate, sort, and organize to reform a 3D tissue mass closely resembling the organs and tissues where the cell types belong (Takeichi, 2011). Recent ESC and iPSC studies have exploited this fundamental cellular principle. Self-organization is a process involving the self-assembly of dissociated cells and the subsequent reorganization into tissue-like structures. Using this approach, termed “dissociation-aggregation approach”, cell progenitors from different lineages can be recombined to form *in vitro* organoids with improved representation of cellular heterogeneity and interactions. In this section, we present a historical overview of the discovery of the self-organizing property of animal progenitors and summarize recent works in human organoids generated based upon this principle. We also review and propose studies by which the self-organizing process could be monitored and regulated.

2.1 REGENERATIVE POTENTIAL OF DISSOCIATED CELLS FROM ANIMAL EMBRYONIC TISSUES AND ORGANS.

2.1.1 Pioneering Experiments in Sponges and Hydroids. In the first series of experiments conducted over a century ago, Henry van Peters Wilson showed that sponges broken down into randomly dispersed single cells were able to reorganize into live sponges (Wilson, 1907). Similar results, detailing the generation of live hydranths, were found in a study performed by Charles Wesley Hargitt (Hargitt, 1915). A similar conclusion was drawn with some essential differences by a later study performed by De Morgan and Drew using different species. In their experiments, the resulting tissue aggregates, or restitution mass, developed the

characterized tissue and cellular architecture of hydranths, e.g. well defined endodermal tubules, the outer perisarc, and the interstitial ectodermal cells, but soon underwent partial disintegration and were never able to give rise to live hydranths (De Morgan and Drew, 1914). Despite these differences, these early experiments sparked enormous interest in the regenerative ability of animal tissues and clearly demonstrated that dissociated cells carried the potential to reform tissues and structures. Tissue cells from different cellular origins, e.g. ectoderm and endoderm, also possessed the ability to attach to one another and undergo cellular rearrangements to achieve an advanced tissue morphology and architecture resembling those found endogenously. Interestingly, Wilson also found that cells from different species of sponges sorted out from each other and only recombined with cells of their own species (Wilson, 1907). This principle is not strictly adhered to in vertebrate cells where chimeric tissues and organs of different species origin could quite frequently be generated.

2.1.2 Regenerative Potential of Embryonic Tissues in Other Animal Species. Following this initial discovery, there remained an important question of whether animal cells generally possess a similar self-organizing ability. Testing of this hypothesis was not feasible until the early 1940s when Johannes Holtfreter discovered a way to conveniently dissociate and re-associate *Xenopus* frog embryonic cells by exploiting the changes in cell adhesion of frog cells to changes in environmental pH (Steinberg and Gilbert, 2004). Thereafter, Aaron Moscona and colleagues employed trypsin, at the time a novel enzymatic tool, for more efficient cell dissociation in avian and mammalian organs and tissues (Moscona, 1957b; Moscona and Moscona, 1952). Culturing conditions for the resultant tissue aggregates however posed additional requirements for more finely regulated nutrient and thermal conditions. This led to the development of more sophisticated culture media and increased precision on the controls for pH and heating to promote prolonged maintenance of tissue masses *in vitro*.

Other advances in culturing techniques such as the advent of rotation cultures (Moscona, 1961) and the use of chorioallantoic membrane as an incubator for organoid tissues (Garber and Moscona, 1964) had facilitated widespread employment of the dissociation-aggregation method and had advanced cellular differentiation of the resulting organoids. Rotation cultures, for instance, involved incubation of trypsin-dissociated cell suspension in a swirling flask, assisting single cells to collide, adhere, and form cell aggregates. It had been used to form mouse embryonic brain cell aggregates displaying a high degree of cellular organization (DeLong, 1970), undergoing the regular biochemical differentiation and expressing enzymes responsible for neural transmission (Seeds, 1971), forming myelination of axons and displaying signs of proliferation and synapse formation (Seeds and Vatter, 1971). Due to concerns that the aggregative features observed in dissociated embryonic cells was an artificial phenomenon created by *in vitro* cultures, a series of studies were carried out aiming to test if dissociated cells could aggregate in an embryonic environment such as the chorioallantoic membrane. In those cases, dissociated embryonic kidney, liver, and skin cells were tested and successfully differentiated as aggregates (Garber et al., 1968; Garber and Moscona, 1964; Weiss and Taylor, 1960).

In another classic embryological experiment, epidermis and neural tube from early amphibian embryos were dissociated and allowed to re-aggregate (Townes and Holtfreter, 1955). The two cell populations sorted out from each other and self-organized into epidermal cells covering the outside of the tissue aggregate containing a neural tube-like structure. Interestingly, there were other studies producing chimeric organoid structures with different animal species or even organs with little relation to each other under normal developmental contexts. For instance,

chimeric experiments were carried out for chick nephrogenic and mouse chondrogenic cells; ultimately concluding that the nephrogenic and chondrogenic cells aggregate according to their tissue types, but disregard their species-specificity (Moscona, 1957a). In another study, mouse skin cells at an advanced stage were found to suppress feather formation in chick-mouse chimeric aggregates, while mouse skin cells from a younger stage appeared to incorporate into the feather structures (Garber et al., 1968). Further, chimeric aggregates of human and mouse cells derived from the same organs (lung, liver, and brain) integrated well with each other—similar to those derived from the same species—suggesting that, between human and mouse, tissue specificity dominates over species specificity (Cassiman and Bernfield, 1974). A similar phenomenon was observed for embryonic chick and rat heart cells, in which chimeric aggregates were formed (Nag et al., 1980). These experiments highlighted the versatility and flexibility of the embryonic and fetal progenitors in chimeric species organ synthesis and inspired more recent experiments that employed the successful integration of embryonic progenitors derived from ESC and iPSC cultures in animal transplantation as a readout for functionality (e.g. Cohen et al., 2016).

2.1.3 Recent Development Using Dissociated Embryonic Progenitors for Organoid Generation. Over the past four to five decades, numerous experiments have continuously demonstrated the tremendous propensity of embryonic and fetal progenitors to self-renew, differentiate, self-organize, and regenerate. In particular, organoid structures have been generated from aggregating embryonic and fetal progenitors derived from virtually all types of embryonic organs and tissues from higher vertebrates including those that are ectoderm (brain, spinal cord, inner ear, submandibular gland, retina, lens), mesoderm (heart, limb bud, kidney), and endoderm (lung, pancreas, liver) derived (Table 1). In one particular example, fragments and aggregates of dissociated cells of embryonic day 13 mouse submandibular gland epithelium were able to self-organize and undergo branching morphogenesis, forming tissues with structural features and differentiation markers characteristic of the intact gland. The study also reported that the self-organization process was sensitive to perturbation by integrin and E-cadherin signaling (Wei et al., 2007). In another well-studied example, dissociated embryonic chick retina cells in rotation cultures were able to reconstitute all the essential retinal layers including differentiating cone and rod cells aligned in rosette structures within the tissue aggregates. These tissue aggregates, so-called rosette spheres, could reach a size of 0.3-0.5 mm and included up to half a million cells. The tissue organization of these rosette spheres was further improved by the inclusion of the retinal pigmented epithelium, which promoted laminar reconstitution and the proper alignment of the different retinal layers (Layer et al., 2001). Many current studies have shifted their focus from simple aggregation experiments to a number of different directions including investigating (1) the identity of the soluble intercellular factors and the intracellular molecular mechanisms that promote the aggregation and the subsequent cell-sorting processes, (2) the role of developmental ages of embryonic and fetal progenitors in organoid formation, (3) the effects of specific dissociation techniques, and (4) the functionality of the resulting tissue aggregates or organoids with more sophisticated biochemical and morphological studies as readouts for function. Documenting these studies is out of the scope of this review, but some of them have been summarized and discussed (Takeichi, 2011).

2.2 AGGREGATION EXPERIMENTS EMPLOYING CELL PROGENITORS DERIVED FROM PLURIPOTENT STEM CELLS.

Given these strong data in animal works and the rich resources of efficient adherent differentiation protocols, recent studies have started to employ dissociated embryonic cell types derived from human ESCs and iPSCs as raw materials for organoid generation. In these studies, ESC and iPSC colonies were differentiated either directly as colonies or after a re-plating step as a monolayer of evenly spread-out, single cells. These differentiating adherent cultures, after reaching the progenitor stages of their respective lineages, were dissociated, dispersed, and re-aggregated to form suspension 3D organoids. In some cases, organoids were derived from progenitors of single germ layers, like in the case of pancreatic (Hohwieler et al., 2017; Kim et al., 2016) and kidney organoids (Takasato et al., 2016; Takasato et al., 2015). In other cases, composite organoids were made from progenitors of multiple germ layer lineages such as liver, cerebral cortical, and intestinal organoids (Schwartz et al., 2015; Shinozawa et al., 2016; Takebe et al., 2013; Takebe et al., 2014; Workman et al., 2017). Many of these organoids have been successfully employed in toxicology studies, disease modeling, and animal transplantation studies. Timed addition of progenitor cell types to composite organoids has allowed increased control over the timing and incorporation of specific cell-cell interactions during organoid generation and differentiation to mimic *in vivo* situations. Cryopreservation of progenitor cell types for these organoids has been successfully performed, thus permitting a consistent and convenient supply of progenitors for organoid formation.

2.2.1 Organoids Derived from Single Germ Layers. ***KIDNEY.*** Mammalian kidneys are derived from the intermediate mesoderm that gives rise to the major kidney progenitors including the ureteric epithelium, metanephric mesenchyme, and the renal stroma. Ureteric epithelium forms the collecting tubes whereas the metanephric mesenchyme forms the nephrons, which include substructures like the proximal and distal tubules and the glomeruli. The mature kidney epithelial structures are surrounded by a renal interstitium containing a vascular network derived from the renal stroma. To generate kidney progenitors, human ESCs were differentiated from the primitive streak stage to form $PAX2^{+}LHX2^{+}$ intermediate mesoderm by manipulation of WNT and FGF signaling (Takasato et al., 2014). Differentiating embryonic kidney cells, resulting from the further differentiation of $PAX2^{+}LHX2^{+}$ intermediate mesoderm progenitors, were dispersed and aggregated to form organoid structures that displayed evidence of development of the ureteric epithelium, the proximal tubules, and the renal vesicles. However, no definitive nephron structures could be identified in these organoids. In a subsequent study, by manipulating the strength and the duration of retinoic acid and WNT signaling, the same group identified a way to preferentially induce ureteric epithelium development over metanephric mesenchyme (Takasato et al., 2016; Takasato et al., 2015). By dissociating progenitors at an earlier time point (day 6 instead of day 18) and optimizing WNT and FGF signaling post-organoid formation, kidney organoids matured to form nephron-like structures including the essential components of collecting ducts, distal and proximal tubules, and glomeruli. Endothelial vascular networks, together with pericyte-like and mesangial-like cells, were also identified and appeared to invade some of the glomeruli. Advanced morphological patterning in terms of the arrangement of the collecting ducts to nephron-like structures and the presence of cortical versus medullary stroma patterning indicated the formation of a complex kidney-like structure. Furthermore, these organoids were tested positive for endocytic function to dextran and for response to nephrotoxic agents like Cisplatin. Comparisons to a range of human fetal tissues

suggested that these kidney organoids resembled first trimester kidney tissues suggestive of their fetal nature and the need for further maturation.

PANCREAS. The pancreas is a glandular organ, which serves dual functions in regulating blood sugar level, by its exocrine glands that secrete insulin and glucagon and in digesting proteins, lipids, carbohydrate, and nucleic acids by excreting enzymes from its endocrine glands. Exocrine (Hohwieler et al., 2017) and endocrine (Kim et al., 2016) pancreatic progenitors have been respectively derived from human ESC and iPSC 2D cultures. These cultures were dissociated and re-aggregated in suspension to form functional and transplantable endocrine and exocrine organoids. Both endocrine and exocrine pancreatic progenitors were differentiated from ESCs and iPSCs via activation of WNT and Activin signaling to prompt a definitive endoderm fate, followed by treatment with retinoid acid, FGF ligand, and BMP inhibitor to generate $PDX1^+$ pancreatic endoderm, a common progenitor for endocrine and exocrine cell types. $PDX1^+$ cells were then skewed towards either exocrine or endocrine progenitors using distinct growth factor and small molecule cocktails.

Pancreatic endocrine cells expressed proprotein convertase 1/3, glucose transporter 1, and the majority of pancreatic hormones such as insulin, somatostatin, and pancreatic peptide, with the exception of glucagon (Kim et al., 2016). Detailed marker profiling, however, suggested that these endocrine cells were still immature. For instance, the authors observed a low expression level of mature β-cell marker NKX6-1 and the coexpression of endocrine progenitor marker MAFB with insulin, indicating that the endocrine cells derived were immature. Upon organoid formation by aggregation of endocrine cells, these cells continued to express pancreatic hormones and the above mentioned functional markers, but, in addition, lost expression of MAFB in $insulin^+$ cells, gained expression of the mature β-cell marker MAFA (Nishimura et al., 2006), and increased expression of glucose sensor genes (*SLC2A1* and *GCK*). All these were indicative of improved β-cell function and maturation. Further, these pancreatic endocrine organoids displayed sensitivity to high glucose concentration and responded by inducing insulin and c-peptide secretion. Functionally, transplantation of pancreatic endocrine organoids prolonged the life span of streptozotocin treated mice, which had their pancreatic islet destroyed by streptozotocin treatment. The mice with organoid transplants displayed a response to high blood glucose and expressed at least one endocrine hormone, c-peptide, in the blood stream. Function of the transplants however gradually became unstable after 2 weeks. Mice survived more than 40 days, but a more long-term survival study was not conducted and ultimate cause of transplant failure was not concluded.

In contrast to endocrine organoids, human pancreatic exocrine organoids formed by aggregation of dissociated exocrine cells contained acinar-like and ductal-like cell types and structures (Hohwieler et al., 2017). Activities for key exocrine enzymes such as carbonic anhydrase, amylase, trypsin, and elastase were detected. Cystic fibrosis patients display increased probability of pancreatitis, pancreatic exocrine insufficiency, and pancreatic cancer. Patient iPSCs carrying *CFTR*[a] mutations were used to derive pancreatic exocrine organoids for modeling cystic fibrosis disease progression. Forskolin is known to induce rapid swelling of control intestinal organoids but not in organoids derived from a cystic fibrosis mouse model

[a]Cystic fibrosis transmembrane conductance regulator (*CFTR*), the gene mutated in cystic fibrosis, encodes a chloride channel protein, a member of ATP-binding cassette transporter superfamily. CFTR mediates fluid homeostasis in the epithelia of organs such as pancreas, liver, intestine, and lung.

(Dekkers et al., 2013). Expectedly, forskolin/IBMX treatment[b] did not induce much swelling in the lumens of human cystic fibrosis patient-derived organoids. Encouragingly, the swelling defect could be ameliorated in a pre-selected set of CFTR correctors and potentiators with known functions in improving CFTR activity, or with chemically modified *CFTR* mRNA that confers high stability and low immunogenicity, demonstrating a proof of principle for these organoids in disease modeling and compound screening. However, upon orthotopic transplantation to a mouse host, the human organoids remained fetal in gene expression, and, because of a lack of functional assays, it was difficult to assess the maturity and purity of the resulting human grafts.

2.2.2 Germ Layer Composite Organoids. INTESTINAL SYSTEM. Attention has often been paid to parenchymal cells in organoid generation. The role of non-parenchymal cells, such as neural cells, mesenchymal cells, and vascular cells, has been well documented in development and tissue functionality (Cleaver and Melton, 2003; Furness, 2012). A recent study, which appreciates the inclusion of these non-parenchymal cell types, generated human intestinal organoids with an intact enteric nervous system (Workman et al., 2017). In this study, dissociated caudal neural crest cells were recombined with human intestinal organoids by low speed centrifugation. Neuronal and glial differentiation was observed in intestinal organoids seeded with neural crest cells. The overall spatial relationship of the resulting composite organoid resembled human fetal or mouse embryonic day 11.5 intestine. These organoids were cultured *in vitro* for 28 days before transplantation. Importantly, the graft displayed neuronal calcium oscillations, contractile activity, and neuromuscular coupling suggestive of a functional enteric nervous system. These grafts were also used to model the phenotype of *PHOX2B* mutations in the etiology of Hirschsprung's disease, a genetic disorder resulting in agangliogenesis of the human bowel.

LIVER. Hepatocytes are the major cell type in the liver, which make up around 80% of the parenchymal mass in the adult organ and display both endocrine and exocrine properties (Si-Tayeb et al., 2010; Zhao and Duncan, 2005). Non-hepatocyte cell types such as cholangiocytes, sinusoidal endothelial cells, Ito cells (hepatic stellate cells), Kupffer cells (tissue macrophages), and pit cells (natural killer cells) constitute the rest of the liver. The liver buds, which are derived from the foregut endoderm and containing the adult liver progenitors, are formed around embryonic day 8.5 in mouse. At embryonic day 9.5, hepatoblasts delaminate from the liver buds to invade the surrounding septum transversum (mesoderm derived), which contains progenitors for non-hepatocyte cell types such as Ito cells and endothelial cells. To recapitulate the *in vivo* cellular heterogeneity of adult liver and to promote the vascularization that is essential for normal liver function and transplantation, Takebe et al. attempted to generate *in vitro* human liver buds by aggregating cell types from two germ layer lineages: hepatic endoderm derived from ESC/iPSC cultures, and from the mesoderm, umbilical vein endothelial cells and mesenchymal stem cells (Takebe et al., 2013; Takebe et al., 2014). A 10: 7: 2 ratio of human $HNF4A^+$ hepatic endoderm, umbilical vein endothelial cells, and mesenchymal stem cells were mixed and resuspended in hepatocyte culture medium. The cells in the suspension aggregated and self-organized to form liver-bud organoids containing an endothelial network and maturing hepatocytes with increased expression of early liver specific genes like *alpha-fetoprotein*, *retinol binding protein 4*, *transthyretin*, and *albumin*. Microarray analyses suggest that the resulting

[b] Forskolin increases the amount of intracellular cAMP, which in turn activates CFTR that mediates fluid secretion into the lumen of small intestinal organoids. IBMX (3-isobutyl-1-methylxanthine) is a phosphodiesterase inhibitor. Because of its effect in inhibiting phosphodiesterase, IBMX may potentiate the accumulation of intracellular cAMP by the action of forskolin.

liver-bud organoids resembled early embryonic liver buds in mouse (embryonic day 10.5 to 11.5). Upon transplantation into mouse brain, connectivity with host vasculatures was quickly observed. Prolonged cultures of transplants up to 60 days led to development of hepatic cord-like structures, which contained cells expressing tight junction proteins and collagens that are normally found along the entire length of the liver sinusoids. Albumin production by day 45 of transplantation was comparable to or surpassed *in vitro* cultures of adult human hepatocytes. Ketoprofen[c] and debrisoquine[d] were used to distinguish human hepatic metabolic functions from mouse hepatic functions since these drugs are metabolized differently in human and mouse livers. Human specific metabolites, upon challenge with ketoprofen or debrisoquine, were found in the transplanted mouse blood serum and urine, suggestive of the presence of functional human liver tissues in the organoids. An additional minimally-invasive mesenteric transplantation model was carried out and the transplanted liver buds promoted survival of mice with ganciclovir-induced liver failure by ~50% at day 30 after transplantation. No specific markers or assays however were used to detect the presence of other non-hepatocyte cell types either under *in vitro* cultures or upon transplantation. These non-hepatocyte cell types play important roles in liver function and pathology. Cholangiocytes, for example, are important for bile metabolism and hepatocyte survival and have been successfully derived from human iPSC cultures (Dianat et al., 2014). For other non-hepatocyte cell types, such as pit cells and Kupffer cells, there are no existing differentiation protocols.

CENTRAL NERVOUS SYSTEM. To mimic *in vivo* brain development, neural constructs (organoids) were generated by aggregating dissociated neural progenitors, mesenchymal stem cells, endothelial cells, and microglial/macrophage precursors (Schwartz et al., 2015). Neural constructs were formed with neural progenitors embedded in polyethylene glycol hydrogels, followed by seeding of endothelial cells and mesenchymal stem cells at day 9 and lastly with microglial/macrophage precursors at day 13 to mimic the *in vivo* recruiting sequence of blood vessels and macrophage after the formation of the neural tube. Timed addition of cell precursors to the initial neural constructs allowed temporal control of the incorporation of different cell types. The resultant neural organoids bore gene signatures for forebrain and hindbrain development and displayed markers for GABAnergic neurons, glutamatergic neurons, and glia. Extensive vascular networks and phagocytic activities were observed in the neural organoids suggestive of functional vascular and macrophage cell types. Machine learning refers to studying and developing algorithms that can iteratively learn from data without being explicitly programmed and make predictions. The neural organoids were employed for machine learning using RNA sequencing datasets with known neurotoxins and control compounds. Subsequent blind trials found that toxicity of 9 out of 10 known compounds could be correctly predicted suggesting that such neural organoids could be used for *in vitro* drug toxicity screens, although the authors admitted that a functional perfused blood-brain barrier could further improve predictability of drug toxicity. The blood-brain barrier is lined by specialized endothelial cells, called brain microvascular endothelial cells that regulate the transport of substances into and out of the brain. To reconstruct a functional adult-like blood-brain barrier, additional cell types are required, such as pericytes and brain microvascular endothelial cells

[c] Ketoprofen is primarily metabolized to ketoprofen-glucuronide by human hepatocytes and metabolized to hydroxyl-ketoprofen by mouse hepatocytes.

[d] Debrisoquine is metabolized to 4-hydroxy debrisoquine by CYP2D6 in human hepatocytes. Mouse liver lacks 4-hydroxylase activity and thus cannot convert debrisoquine into its 4-hydroxy metabolite.

(Lippmann et al., 2013). These cell types have been derived from human ESC and iPSC cultures (Lippmann et al., 2012; Orlova et al., 2014).

2.2.3 Monitoring and Regulating the Self-organization Process to Facilitate Organoid Formation. We envisage more widespread usage of the dissociation-aggregation method in future studies, especially for organ systems whose full functionality depends on the incorporation of multiple germ layer lineages. To improve this method, we propose timed and sequential incorporation of tissue- and organ-specific cell progenitors to better mimic the events occurring during embryonic development (Figure 2). Self-organization of the aggregating cells during organoid formation can be broken down into steps of temporally-overlapping and interrelated processes that could be monitored and regulated. These include the chemotactic movement of the aggregating cells (Chen et al., 2007), the cell surface interactions to matrices and to neighboring cells (Takebe et al., 2015), the cell-sorting process, the formation of the apical-basal orientation of individual cells, and the process of lumenogenesis (Odenwald et al., 2017; Taniguchi et al., 2015) (Figure 3). Time-lapse imaging of aggregating cell populations labeled transiently with fluorescent dyes or permanently with genetically-engineered reporters could allow tracking of the kinetics of organoid formation, whether they form under certain conditions or not, and, if they do form, how fast they form, and the dynamic changes of their shape along the time course of their formation. The speed of condensation and the changes in the shape of the forming organoids could be monitored by quantitative analyses (Takebe et al., 2015). Additionally, pharmacological interventions can be applied to regulate the speed of aggregation. Takebe et al. found that manipulation of myosin II activity controlled the collective movement of cells during the aggregation process (Takebe et al., 2015). Mesenchymal stem cells within the initial aggregating mix were identified as a key cell population that was essential to drive the aggregating process (Takebe et al., 2015). By closely monitoring the structural parameters of the forming organoids, such as their diameter and morphology, Arora and colleagues were able to promote the yield of successful intestinal organoids using an automated micro-peptide aspiration and release system (Arora et al., 2017). More studies in these areas are urgently needed to translate findings from organoid monitoring into promoting the successful formation of organoids.

3 UTILIZING DESIGNER MATRICES AND SCAFFOLDS FOR ORGANOID FORMATION

3.1 DESIGNER MATRICES AND MATRIX PARAMETERS. When considering the minimal elements for forming a functional organ or tissue, one must consider not only parenchymal cells and the less-represented and essential cell types like immune, vascular, and neural cells, but also the secreted ECM framework and its associated growth factors, cell interacting molecules, enzymes, and extracellular vesicles that produce the necessary physical and biochemical signals to support organ and tissue maintenance, growth, and morphogenesis. To mimic the physical and biochemical properties of *in vivo* cellular microenvironment, designer matrices (Gjorevski et al., 2014), either derived from natural sources or artificially synthesized with physical and biochemical properties specially designed for a certain cell type can be employed. Controls exercising on organoid formation can be greatly facilitated by incorporating designer matrices. For example, micropores present in hydrogels and scaffolds would allow organoid formation at a defined shape and size. The physical and biochemical properties of designer matrices can be spatially and/or temporally controlled to enable stem cells to self-organize, and this topic has been elegantly reviewed (Gjorevski et al., 2014). Together with

advanced 3D printing technology, studies can be conducted to fabricate designer matrices with controlled nano- and micro-structures to standardize and improve organoid formation. We propose that by studying the endogenous composition and dynamics of ECM expression during the development of animal and human organs and tissues and empirically testing the requirements of these ECM and other tailor-made matrices in *in vitro* cultures, one would be able to determine the minimal and essential elements and signals from the ECM that are required for the cellular activity, organization, and differentiation of organoid cultures.

A number of parameters dictate the ability of matrices to modulate organoid formation. These parameters include stiffness, composition, topology (or geometry), degradability, the ability to bind growth factors, and the capacity to modulate growth factor activities. Regulating these parameters may prove advantageous to promoting organoid formation, standardizing organoid production, and moving organoid technology to clinics. Organoids are currently cultured either matrix-free or by embedding them in matrices like Matrigel. Matrix-free suspension organoid cultures display superb nutrient diffusion, but lack certain physicochemical supports, resulting in organoids that may clump and causing lower yields, and may develop necrosis within the core. Matrix provides an initial guidance to the aggregating cells and serves as a subsequent physical support and constraint for organoid formation. Matrigel and collagen type I are the most commonly used matrices in organoid cultures derived from ESCs, iPSCs, and adult stem cells. However, there are few studies investigating the precise roles of these matrices in supporting organoid formation. On top of this, the animal origin and lot-to-lot variation of Matrigel hinders the use of the derived organoids in clinical applications. Fully synthetic or recombinant matrices are chemically defined and can be standardized for organoid production and better facilitate downstream clinical applications. Well-defined matrices, such as those incorporating synthetic ECM peptides with artificial hydrogels, allow precise control of the cellular microenvironments.

Matrix stiffness is an important parameter regulating cell behavior. Matrix stiffness reflects the resistance that a cell experiences when it deforms the matrix, and it can be measured by the elastic constant (Young's modulus, presented as Pascal). Cells sense matrix stiffness through mechanoreceptors, such as integrins (Humphrey et al., 2014). It was found that a stiffer hydrogel (1.3 kPa) supported intestinal stem cell expansion, whereas softer hydrogel (approximately 190 Pa) promoted differentiation and intestinal organoid formation, suggestive of a role played by matrix stiffness in regulating cell proliferation versus differentiation in 3D environments (Gjorevski et al., 2016). Earlier studies also revealed an important function of matrix stiffness in lineage specification of mesenchymal stem cells in 2D (Engler et al., 2006) and 3D environments (Huebsch et al., 2010) and ESCs in 2D environments (Chowdhury et al., 2010). As a prime example, precise temporal control of matrix stiffness can be achieved by synthesizing a composite with specific proportions of degradable and non-degradable synthetic hydrogels, thus providing additional guidance to stem cell renewal and differentiation as shown in intestinal organoid cultures (Gjorevski et al., 2016).

3.2 CHARACTERIZATION OF ECM IN IN VITRO CULTURES AND DURING DEVELOPMENT. ECM composition and architecture is under constant remodeling during normal development (Daley et al., 2008) and ESC differentiation. Data from human embryonic liver development demonstrated dynamic expression pattern of integrins and ECM components (Couvelard et al., 1998). During embryoid body formation from human ESCs, the expression of fibronectin was spatiotemporally correlated with the expression of a definitive endoderm marker GATA4 (Taylor-Weiner et al., 2013). A reverse correlation was found with the expression of a

pluripotent marker NANOG. Directed differentiation of ESCs to definitive endoderm produced fibrillar fibronectin whereas other lineage differentiation or ESC cultures produced punctate fibronectin. Kanninen et al. have recently discovered that integrin expression was dynamic during hepatic differentiation of human ESCs and iPSCs (Kanninen et al., 2016). The expression of laminin-511/521-specific integrins increased during definitive endoderm induction and hepatic specification. They showed that laminin-511 and laminin-521 promoted hepatic specification from human definitive endoderm cells (Kanninen et al., 2016). Recombinant laminin-511 and laminin-521 would presumably provide an optimal biochemical cue as a designer matrix to assist generation of liver organoids.

To understand the dynamic changes in ECM, temporal expression studies need to be carried out to examine the changes in ECM molecule expression during consecutive developmental stages. Studying the secretome of a cell population that includes growth factors, extracellular matrices, extracellular proteinases, and enzymes may prove to be extremely useful. For example, transcriptomic analyses have been performed to determine the changes in secretome across several early kidney developmental stages (Martinez et al., 2006). More advanced technology involving the use of automated and high-throughput tandem mass spectrometry (MS) can be employed to identify the secretome and the proteome of the ECM of cultured cells under various experimental conditions and of *in vivo* tissues across developmental stages (Byron et al., 2013; Ngounou Wetie et al., 2013). Steps such as sample or peptide fractionation and liquid chromatography (LC) prior to MS can help reduce sample complexity and ease the identification of ECM components. Such approaches had been successfully used to identify tens to hundreds of ECM components from tissues ranging from eyes to mammary glands to cartilages (Byron et al., 2013). Solid-state NMR spectroscopy and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) have been suggested for the compositional analysis of natural ECM and bioengineered tissues (Schiller and Huster, 2012). Low abundant proteins can be detected directly with high-resolution MS or a coupled LC and LC-MS/MS system (i.e. ESI-Q-TOF MS, FT-ICR-MS) (Personal communication with Dr. Andrzej Ciechanowicz). To investigate a more dynamic biological system, as occurs in development, quantification of the relative or absolute amount of particular ECM and secretome components can be achieved by MS using isotope labeling or other label-free strategies. Key ECM and secreted biologically-active protein components identified from secretome assay could be tested for functionality in promoting organoid formation and development via loss-of-function experiments or ectopic administration. Recently, an ECM array platform has been developed to empirically examine the composition of ECM molecules permissive for definitive endoderm differentiation towards the liver and pancreatic lineages (Braga Malta et al., 2016). Coupling array studies with the protein expression data from MS assays will provide valuable information for formulating designer matrices in organoid formation.

3.3 APPLICATIONS OF DESIGNER MATRICES. To incorporate the dynamic changes in ECM composition into organoid differentiation protocols, one may passage dissociated organoid cells at defined time points onto predesigned matrices and scaffolds or may incorporate relevant ECM components into a bio-inert scaffold or hydrogel with degradable matrices, as described previously (Gjorevski et al., 2016). The same study found that a synthetic hydrogel, polyethylene glycol, did not support organoid formation (Gjorevski et al., 2016). Polyethylene glycol cannot interact with cells; it only provides physical support. This finding indicates that additional biochemical signals generated by the biologically-active matrices were critical for organoid formation. Biologically-active matrices can generate signals either by

directly interacting with cell membrane receptors such as integrins or by binding to growth factors. Many ECM components have integrin-binding domains and/or growth-factor-binding domains, which can be incorporated into designer matrices. It is well-known that the ECM binds and influences the activities of growth factors (Rozario and DeSimone, 2010). More sophisticated design of matrices can be used to create concentration gradients of growth factors, thereby guiding morphogenetic events. Growth factors can also be conjugated on matrices to sustain their releases to cells during prolonged organoid cultures (Lutolf and Hubbell, 2005).

To achieve endogenous matrix architecture, a recent study used multi-photon excited 3D printing technology to produce an ECM scaffold that mimics the developing mouse heart tissues with submicron resolution (Gao et al., 2017). The scaffold was seeded with component cell types of developing heart derived from human ESC and iPSC, such as cardiomyocytes, endothelial cells, and smooth muscle cells. The resultant so-called cardiac muscle patches, although much smaller in size than regular organoids, tested positive for cell engraftment and increased cardiac function in a mouse model for cardiac infarction. In this study, however, only fibronectin was used. Incorporation of multiple relevant ECM molecules mimicking the endogenous composition of ECM in the heart and other organ systems will enhance the applicability of this 3D scaffold printing technology.

Collectively we propose that designer matrices fabricated based on the understanding of the dynamic changes in ECM parameters during *in vivo* organ and tissue development and robust empirical testing could maximize the self-organizing capacity of cells and provide an optimal environment for human organoid formation.

4 PROMOTING THE MATURATION OF ORGANOIDS

In vitro organoid cultures have thus far failed to produce fully-mature cell types. Intestinal, gastric, lung, kidney, and cerebral organoids derived from human ESCs and iPSCs exhibit immature phenotypes, resembling human fetal organs (Camp et al., 2015; Dye et al., 2015; Finkbeiner et al., 2015; Hohwieler et al., 2017; McCracken et al., 2014; Takasato et al., 2015). To promote the maturation of *in vitro*-derived organoids, maturation factors need to be introduced and culturing conditions must be adjusted at subsequent stages of differentiation to drive the transition of fetal organoids into more adult-like phenotype. Narrowly, "maturation factors" could refer to proteins or chemical factors secreted from cells that promote the maturation of fetal tissues towards more adult-like tissues. In a broader sense, they could refer broadly to factors ranging from cell-cell interacting factors, circulating hormones, microbial-derived factors (Avior et al., 2015), electrical signals to other unknown factors that could promote the same process for specific cell types. There is ongoing debate on how maturation of embryonic tissues is achieved. One hypothesis suggests that tissue maturation is driven by the induction of adult stem cells in fetal tissues. An alternative hypothesis suggests that authentic tissue-specific fetal stem cells, distinct from adult stem cells, exist during development and contribute to tissue maturation. In this section, we review studies and propose a number of approaches with which maturation of organoids could potentially be achieved. Furthermore, we review current studies that attempt to promote the *in vitro* maturation of organoids with variable successes.

4.1 ORGANOID MATURATION UPON TRANSPLANTATION. There are concerns that organoids derived from human ESC and iPSC, even after prolonged cultures, might be unable to mature *in vitro* to become adult-like tissues. However, that transplantation into animal tissues in many cases induces maturation of human organoids (Cho et al., 2017; Finkbeiner et al.,

2015; Takebe et al., 2013; Watson et al., 2014; Workman et al., 2017) suggests that functional maturation of *in vitro* organoids could be achieved. For instance, transplantation into the kidney capsule of mice promoted the maturation of both the intestinal and the enteric nervous system of human intestinal organoids (with neural crest-derived enteric neurons) after engraftment for 6 to 10 weeks (Workman et al., 2017). Highly mature intestinal tissues, with villi and crypts containing functional intestinal stem cells, were formed *in vivo*. Enteric neurons and glia were organized into ganglionic structures in close proximity to the submucosal and myenteric layers of smooth muscle fibers. Nitric oxide synthase expression was detected upon transplantation, another sign of maturation of the enteric nervous system. One possible explanation is that vascularization of organoid structures brings in by the blood stream active components like hormones and serum proteins that could assist a general tissue maturation process. This notion is supported by the enhanced maturation of human intestinal and liver organoids upon ectopic transplantation into adult tissue sites away from their organs of origin, such as to kidney capsule and brain respectively when compared with *in vitro* cultured organoids (Finkbeiner et al., 2015; Takebe et al., 2013). Another possibility is that this transplantation-induced maturation could be due to the direct contact of immature organoid cell types with an adult environment. For instance, adult stem cell niche factors, present in the *in vivo* tissues, may play a role during the maturation process (section 4.2).

4.2 ADULT STEM CELL NICHE FACTORS AS POTENTIAL MATURATION FACTORS. Adult stem cells (also called tissue stem cells) reside in a tissue-specific environment, called the stem cell niche. A stem cell niche maintains the self-renewal of adult stem cells and is comprised of ECM, soluble factors, and in some cases niche support cells (Rojas-Rios and Gonzalez-Reyes, 2014). Organoids have been derived from adult stem cell-containing tissues, such as the intestinal crypts (Sato et al., 2009), the colonic crypts (Sato et al., 2011), the gastric glands (Barker et al., 2010; Bartfeld et al., 2015), the biliary ducts (Huch et al., 2013b), and the pancreatic ducts (Boj et al., 2015; Huch et al., 2013a). To demonstrate the self-renewal and multipotency of adult stem cells, organoids have also been clonally derived from single proliferative adult stem cells isolated from a number of mouse and human organs and tissues including the small intestines (Sato et al., 2009), the colons (Sato et al., 2011), the gastric pyloric glands (Barker et al., 2010), the gastric corpus tissues (Bartfeld et al., 2015), the pancreatic ducts (Huch et al., 2013a), the biliary ducts (Huch et al., 2013b), the liver (Huch et al., 2015), the lungs (Kumar et al., 2011; McQualter et al., 2010), the prostate gland (Chua et al., 2014; Karthaus et al., 2014), the mammary glands (Jamieson et al., 2016; Shackleton et al., 2006), the salivary glands (Nanduri et al., 2014), the retina (Tropepe et al., 2000), and the fallopian tubes (Kessler et al., 2015). Adult stem cell-derived organoid structures were formed either via the curling up of adult stem cell-containing tissue fragments or via cell division of single stem cells into closed cyst-like structures. Most of the clonally derived adult stem cell organoids were embedded within laminin-rich Matrigel and comprised only of epithelial cells without the presence of stromal and mesenchymal cell types. In rare cases when intestinal tissue fragments that contained stroma were cultured, the derived organoids would consist of polarized epithelial cells surrounded by myofibroblasts and the epithelial compartment developed both crypt-like structures and villus-like protrusions into the lumens (Ootani et al., 2009). Adult stem cell-derived gastric organoids showed higher expression of differentiated markers for glandular cell types such as mucous neck, chief or endocrine cells (*Muc6*, *Pgc*, *Gif*, *Chga*, *Chgb*, *Sst*, and *Gast*) (Fernandez Vallone et al., 2016). Transcriptomes of organoids derived from adult stem cells clustered with their corresponding adult cells based on gene expression profiling studies.

While global gene expression profiling by RNA sequencing revealed that human gastric organoids derived from human ESCs/iPSCs resembled human fetal instead of adult stomach tissues (McCracken et al., 2014). There is no single study to our knowledge that has directly compared the transcriptomes of organoids derived from human ESCs/iPSCs and adult stem cells.

Adult stem cell-derived organoids are cultured in conditions that attempt to mimic the paracrine signals and the ECM components provided by their corresponding stem cell niches. Identification of these culturing parameters and conditions may prove beneficial in identifying cues to control the expansion and maturation of organoids derived from human ESCs and iPSCs. The commonly used niche factors in mouse intestinal organoid cultures are EGF, Noggin, and R-spondin 1 (abbreviated as ENR condition). ENR condition was sufficient to maintain mouse intestinal stem cell self-renewal and promoted differentiation to all intestinal epithelial lineages. To further promote self-renewal and suppress differentiation of stem cells, two small molecules, a glycogen synthase kinase 3β inhibitor CHIR 99021 and a histone deacetylase inhibitor valproic acid, have been employed (Yin et al., 2014). Under this condition, morphology of organoids became homogenous and formed elongated crypt (budding) structures. Intestinal stem cells (Lgr5-GFP) were also found throughout the organoids. In contrast, manipulation of Wnt and Notch signaling directed differentiation of organoids into specific lineages such as enterocytes, goblet cells or Paneth cells (Yin et al., 2014).

Other types of adult stem cell organoids required not only the ENR condition but also additional factors for self-renewal and growth. Some of these additional factors may suppress differentiation. For example, on top of ENR, mouse colon organoid cultures required exogenous Wnt3A (Sato et al., 2011); human intestinal and colon organoid cultures Wnt3A, gastrin, nicotinamide, a TGFβ inhibitor A-83-01, and a p38 inhibitor SB202190 (Sato et al., 2011); mouse gastric organoid cultures Wnt3A and FGF10 (Barker et al., 2010); and human gastric organoids Wnt, gastrin, FGF10, and A-83-01 (Bartfeld et al., 2015). To induce or control the rate of differentiation, some of the niche factors were removed or their concentrations adjusted. Mouse colon organoids and human intestinal and colon organoids under growth conditions did not produce differentiated cells. To induce differentiation, Wnt3A, A-83-01, and SB202190 needed to be withdrawn (Sato et al., 2011). Also, mouse gastric organoids under growth condition expressed gastric epithelial markers but lacked markers of pit and enteroendocrine lineages (Barker et al., 2010). Reduction in Wnt3A concentration resulted in the formation of pit cells, mucus neck cells, and enteroendocrine cells. Differentiation of human gastric organoids could be controlled by addition of nicotinamide for gland-type organoids and withdrawal of Wnt for pit-type organoids (Bartfeld et al., 2015).

In the normal adult liver, stem cells have been identified in the pericentral (Wang et al., 2015) and periportal regions (Miyajima et al., 2014). These stem cells can give rise to both hepatocytes and biliary epithelial cells (cholangiocytes). EPCAM is a marker for these liver stem cells (Schmelzer et al., 2007). $EPCAM^{+}$ cells from bile ducts of adult human liver produced organoids in the presence of EGF, R-spondin 1, FGF10, nicotinamide, HGF, a TGFβ inhibitor A-83-01, and a cAMP pathway agonist Forskolin (Huch et al., 2015). *In vitro* hepatocyte differentiation of the organoids required BMP7 treatment followed by the withdrawal of R-spondin 1, FGF10, nicotinamide, and Forskolin and the addition of FGF19, DAPT, and dexamethasone. The resulting cells exhibited hepatocyte morphology, expressed high levels of hepatocyte markers and performed liver functions, such as albumin secretion, CYP3A4 activity, LDL uptake, glycogen storage, bile acid salt secretion, and ammonia detoxification. Upon transplantation, human liver organoid cells also became hepatocyte-like cells and produced

human albumin, though the level was lower than that produced by transplanted primary human hepatocytes.

Pancreatic ductal organoids have been generated from duct fragments or ductal cells of normal mouse pancreas using ENR, FGF10, nicotinamide, and gastrin; human pancreatic organoids additionally required Wnt3A, A-83-01, and prostaglandin E2 (Boj et al., 2015; Huch et al., 2013a). These organoids exhibited budding structures with an enriched ductal cell population, but they were devoid of acinar and endocrine lineages (Boj et al., 2015). Following orthotopic transplantation into the tail region of the mouse pancreas, both mouse and human organoids developed ductal structures evidenced by the expression of CK19, but the presence of other lineages was not reported (Boj et al., 2015).

Regeneration of ectoderm-derived tissues has enormous value in treatments of degenerative diseases such as age-related macular degeneration and Alzheimer's disease. By colony-forming assay, retinal stem cells were identified from the ciliary margin in the adult mouse eye (Tropepe et al., 2000). These cells were pigmented cells that could clonally proliferate and give rise to spheres *in vitro*. FGF2 signaling promoted their proliferation and colony forming ability. A single pigmented cell from the ciliary margin generated in 7 days a large sphere consisting of 13,000 pigmented and non-pigmented cells. They proliferated as spheres and acquired the expression of CHX10 and nestin. Under differentiation conditions, the cells in spheres differentiated into MAP2-expressing neuronal cells and GFAP-expressing glial cells. Some undifferentiated nestin-positive cells remained in the center of spheres. When the cells from spheres were cultured in 2D under the same differentiation condition, they became flattened pigmented cells without neural markers.

Collectively, the use of adult stem cells in organoid generation has been mostly from endoderm-derived epithelial tissues (intestine, colon, stomach, liver, pancreas, lung, and prostate), a few ectoderm-derived epithelial tissues (mammary gland, retina, and salivary gland), and mesoderm-derived epithelial tissue (fallopian tubes) (summarized in Table 2). There is no current attempt to bridge our understanding on the culturing conditions for adult stem cell-derived and ESC- and iPSC-derived organoid cultures. Owing to the functions of stem cell niches in controlling proliferation and differentiation of adult stem cells, conditions mimicking the paracrine signals and the ECM components provided by their corresponding stem cell niches would be ideal for adult stem cell-derived organoids. However, only the design of intestinal organoid cultures was based on its niche. Other types of organoid cultures were developed by modifying the existing intestinal organoid conditions. Essential niche factors for other organ and tissue systems are not well characterized.

4.3 EXPLORING DEVELOPMENTAL CUES IDENTIFIED DURING NORMAL FETAL TO ADULT TRANSITION. Recent studies on a number of human fetal tissues suggest the existence of fetal stem cells, which display distinct features and differentiation potentials from adult stem cells. For instance, stem cell populations have been identified and isolated from a number of human fetal tissues like neuroretina (Zhou et al., 2015), kidney (Da Sacco et al., 2017), heart muscle (Leung et al., 2015), skeletal muscle (Alexander et al., 2016), pancreas (Bonfanti et al., 2015), intestine (Fernandez Vallone et al., 2016), and brain (Kallur et al., 2006; Kim et al., 2006), which are proliferative, self-renewing, and maintain tissue specific gene signatures when cultured *in vitro*. For instance, fetal enterospheres can be established from proliferative progenitors derived from human fetal intestinal tissues at gestational week 10 and the correspondingly-aged mouse fetal intestinal tissues at embryonic day 16.5 (Fordham et al., 2013). These fetal enterospheres could be passaged for an extended period of time (up to 2

months for human and 2 years for mouse), were cultured in specific medium conditions distinct from adult organoids (ENR condition with the addition of PGE2 for human and ENR medium alone for mouse), and exhibited a smooth spheroid morphology in contrast to those organoids derived from Lgr5+ adult stem cells, which had budding protrusions on their surfaces. Apart from morphology, there are also distinct gene signatures that define adult stem cells and fetal progenitors. For instance, *LGR5* specifically labeled adult stem cells in the intestine and the stomach (Barker et al., 2010; Huch et al., 2013b; Sato et al., 2009) but was expressed at relatively low levels in fetal tissues (Fernandez Vallone et al., 2016). Instead, fetal tissues were characterized by high expression of genes such as *TROP2* and *TNFRSF19* (Fernandez Vallone et al., 2016). So far, the differences found in organoid morphologies, culturing requirements, and gene expression profiles have been mainly characterized in adult and fetal intestines. Further investigation needs to be performed on other developing and adult organ systems to validate the generality of this phenomenon. These results so far support the notion that immature proliferative progenitors exist in mammalian fetal tissues and are developmentally distinct from adult stem cells in terms of the organoid structures they derive, their gene expression profiles, and their signaling requirements.

Studying the natural fetal to adult transition of embryonic tissues bears enormous implications on how to promote terminal differentiation of ESC- and iPSC-derived tissue-specific cell types, which very often suffer from an insufficient degree of cellular maturation. It is therefore imperative to promote studies in mammalian species to understand the signaling events and gene expression dynamics during the normal developmental transition of fetal tissues towards adulthood. Studies focusing on analyzing the stage-wise morphological and transcriptomic changes of fetal and perinatal tissues are particularly useful in identifying tissue-specific and functionally-relevant gene markers to label and isolate stem cell and progenitor populations from differentiating human ESCs and iPSCs. By performing a three-way comparison between the transcriptomes of human intestinal organoids derived from pluripotent stem cells and human fetal and adult intestinal tissues, Finkbeiner et al. identified *OLFM4* as a marker for intestinal tissue maturation, with enhanced specificity compared to the traditional adult stem cell marker *LGR5* (Finkbeiner et al., 2015). In another study, microarray datasets of developing mouse heart tissues were collected and their transcriptomes were analyzed to reconstruct a gene regulatory network involved in heart maturation (Uosaki et al., 2015). Pathway analyses were employed to identify key pathways and upstream regulators activated at subsequent embryonic and adult stages. For example, the authors identified that the peroxisome proliferator-activated receptor (PPAR) pathway became increasingly active during subsequent developmental stages in the heart and therefore correlated to cardiac tissue maturation. In a similar study, matched human fetal atrial and ventricular heart tissues at first and second trimester stages corresponding to 7, 15, and 20 weeks of gestation were analyzed using microarrays (van den Berg et al., 2015). Ventricular- and atrial-specific gene signatures were identified. Interestingly, genes encoding histone H1 variants such as *HIST1H3I*, *HIST1H2BM*, and *HIST1H2A1*, and those for the ECM collagens such as *COL1A2*, *COL2A1*, and *COL15A1* were downregulated and upregulated respectively in second trimester heart tissues as compared to those from the first trimester, suggestive of a developmental change in gene expression pattern during human heart tissue maturation. In order to translate these transcriptomic studies into useful information for *in vitro* organoid cultures, gain- and loss-of-function experiments can be carried out in animals to identify key molecules and pathways regulating the maturation of specific tissues.

4.4 CURRENT ATTEMPTS ON PROMOTING ORGANOID MATURATION. A number of studies have attempted to promote *in vitro* maturation in human organoids. One approach aims to generate reporter lines that allow isolation of the rare adult stem cell populations from differentiated human ESC and iPSC cultures. Using zinc finger genome editing technology, Forster et al. engineered a GFP reporter insertion into the endogenous locus of the intestinal adult stem cell marker *LGR5* (Forster et al., 2014). By flow cytometry-assisted sorting of teratoma tissues derived from this knock-in human iPSC line, the authors were able to isolate $LGR5^+$ progenitors that could form organoids similar in culturing conditions and differentiation potentials to intestinal organoids derived from $LGR5^+$ adult stem cells. They additionally found that maintenance of these adult stem cell-like derived organoids required both WNT and Notch signaling. A second approach aims to identify conditions for maturation either empirically or by inference from culturing conditions of adult stem cells. For instance, it was found that maturation medium containing T3 hormone significantly promoted the maturation of *in vitro* human ESC- and iPSC-derived cardiomyocytes, which corresponded to second trimester human heart tissues (van den Berg et al., 2015). The maturation status of those cardiomyocytes not treated with the maturation medium remained at first trimester. One key feature of primate cerebral cortex is the presence of gyrification. Human cerebral organoids generated so far lack cortical folding. A recent study suggests that enhanced AKT signaling caused by PTEN mutation promotes formation of folds in human cerebral organoids (Li et al., 2017). It is unknown whether *in vitro* activation of AKT pathway may promote maturation of cerebral cortical organoids. Many adult stem cell organoid markers are WNT targets, suggesting that adult organoids likely have high WNT signaling and activation of WNT signaling could promote fetal-to-adult transition of fetal organoids. Indeed, it has been shown that inclusion of Wnt3A ligand in cultures for fetal progenitor derived enterospheres could promote their maturation into adult stem cell-like derived organoids (Fordham et al., 2013). Some other studies have started to exploit whether ECM and bioengineered scaffolds may provide cues for maturation. For instance, salivary gland spheres were expanded in Matrigel and then differentiated into organoids in a mixture of Matrigel and collagen (Nanduri et al., 2014). Another recent study reports improved maturation and engraftment of lung organoids by using a microporous poly(lactide-co-glycolide) scaffold (Dye et al., 2016). These experiments strongly suggest that, to a certain extent, maturation of organoids is achievable *in vitro*.

5 BIOMEDICAL APPLICATIONS AND PERSPECTIVES OF NEXT GENERATION HUMAN ORGANOIDS

A number of excellent reviews have recently been published on the usage of organoids in pharmaceutical and clinical research. Here, we aim to highlight the potential improvements on organoid synthesis that our review discusses and propose studies that will benefit a range of biomedical applications including disease modeling, drug discovery, and tissue transplantation.

5.1 MIMICKING THE COMPLEX CELLULAR HETEROGENEITY OF UNDERSTUDIED ORGANS AND TISSUES. So far, most of our discussion has been limited to internal organs and brain tissues. Organoids or 3D cultures for many other tissues, especially those that involved the incorporation of ectoderm-derived cell types, such as inner ears, cranial ganglia, skin, limbs, and eyes, have been less commonly attempted. The major difficulty associated with synthesizing organoids for these tissues is the lack of robust differentiation approaches for the co-induction of specific progenitors in the right proportions from the same or different germ layers. For instance, the mammalian ear, which includes the outer, middle, and

inner ear, is derived from cell types generated from all three germ layers. Defects in any of the cell components could lead to conductive or sensory hearing loss. Another example is the ganglionic structures that develop in the head region, such as the trigeminal and vestibulocochlear ganglia, the latter of which contributes to the inner ear. Disorders like sensory or age-related hearing loss and migraine arise from malfunctions of these ganglia. These ganglia are composed of sensory neurons, which are derived from cranial placodes, and glial cell types, from neural crest. Both cranial placodes and neural crest are ectoderm derived but their induction regimes in ESCs and iPSCs differ dramatically (Dincer et al., 2013; Leung et al., 2013; Leung et al., 2016; Menendez et al., 2011; Mica et al., 2013). Neurovascular networks for internal organs are composed of autonomic neurons, endothelial cells, and vascular smooth muscle cells. The introduction of an intact neurovascular network into organoids for internal organs will allow more precise disease modeling, for instance, for Hirschsprung disease (Workman et al., 2017) or other genetic disorders with a disrupted neurovascular development. A recent study found that proper differentiation of hESC-derived autonomic neurons was dependent on contacts with endothelial cells and vascular smooth muscle cells and that the co-cultures of all three cell types led to the development of an organized neurovascular network (Acevedo et al., 2015). This again demonstrates that physiologically relevant progenitors can self-organize to reform endogenous tissue-like structures *in vitro*.

5.2 ENHANCING SAFETY, EFFICACY, AND COST-EFFECTIVENESS OF DRUG TESTING. Drug development is an expensive (1.78 billion US dollars for a new molecular entity) and lengthy (average 13.5 years) process (Paul et al., 2010). The biggest challenge facing the pharmaceutical industry is the high attrition rate in drug development. The product failing rates in the United States, Europe, and Japan between 1990 and 2004 had drastically increased, approximately 75% at preclinical phase, 70% at phase I, 55% at both phase II and phase III (Ledford, 2011). A recent study analyzing drug attrition from four major pharmaceutical companies shows that drug toxicity was the primary cause of attrition at preclinical phase and clinical phase I (Waring et al., 2015). The likelihood of final approval counting from the start of clinical phase I was only about 10% (Hay et al., 2014; Kola and Landis, 2004). The main causes of attrition were lack of efficacy and safety, each contributing to approximately 30% of failures (Kola and Landis, 2004). During 2007-2010, 66% of phase III submission failures were attributable to lack of efficacy, particularly in the therapeutic areas of oncology and neurodegeneration, and 21% of failures were caused by safety issues (Arrowsmith, 2011). Moreover, drug withdrawal from the market due to toxicology has a remarkable impact on the pharmaceutical industry and also on patients. Adverse drug reactions are serious problems and considerably increase morbidity, mortality, and health care costs (Pirmohamed et al., 2004). Drug-induced liver injury was the most common reason for the withdrawal of clinical drugs from the worldwide market during 1953-2013 (18% of all withdrawals) (Onakpoya et al., 2016). Another study reported the top three reasons for the post-marketing withdrawal in the EU during 2002-2011 were cardiovascular toxicity, hepatotoxicity, and neurotoxicity (McNaughton et al., 2014). To improve R&D productivity and quality of health care, obtaining human proof-of-concept data early in drug development and identifying risks at the preclinical phase are crucial.

An ideal *in vitro* model for preclinical drug testing should mimic human pathophysiology to promote accurate prediction of drug efficacy and toxicity. Next generation human cell-derived organoids generated by the incorporation of the correct cellular heterogeneity, maturation factors, and designer matrices would bear a closer resemblance to the *in vivo* tissues and organs than conventional 2D cultures and 3D organoids. They would have close to native cellular

composition and could better predict drug efficacy and toxicity at a tissue or organ level. Also many drug-induced injuries are not caused by direct effects on parenchymal cells but rather caused by immune responses or non-specific systemic effects from non-parenchymal cell types. For instance, some compounds show liver toxicity *in vivo* but are not toxic to hepatocytes or only at extremely high concentrations, indicating that *in vitro* testing using only hepatocytes is not adequate to predict hepatotoxicity (Godoy et al., 2013). Non-parenchymal cells in the liver, such as Kupffer cells and hepatic stellate cells, may contribute to inflammation during drug-induced liver injury (Godoy et al., 2013). Liver organoids with Kupffer cells or hepatic stellate cells would predict such hepatotoxicity more accurately than those with primary human hepatocytes, which is the current gold standard for *in vitro* drug testing. Hepatic stellate cells also contribute to the development of liver fibrosis. Recently human hepatic organoids derived from HepaRG and primary human hepatic stellate cells have been used to evaluate drug-induced liver fibrosis (Leite et al., 2016). Liver organoids derived from human ESCs/iPSCs with normal genotypes would be superior to HepaRG, which is a human hepatocellular carcinoma-derived hepatic progenitor cell line. Organoids derived from human iPSCs of different genetic backgrounds would help develop personalized toxicology, which will pave the way to precision medicine. A study successfully reproduced individual differences in drug metabolism capacity and responsiveness by using a panel of human iPSC-derived hepatocyte-like cells (Takayama et al., 2014). Liver organoids with bile duct-like structures formed by including cholangiocytes would predict drug-induced cholestasis. 3D hepatocyte spheroids derived from human ESCs and iPSCs exhibited increased expression of drug metabolizing enzymes and transporters and increased sensitivity to tested drug compounds, compared to traditional hepatocellular carcinoma-derived cell lines like HepG2 cells (Takayama et al., 2013). Similarly, neurotoxicity has contributed to attrition of drug candidates. A recent study by (Schwartz et al., 2015) has constructed neural organoids using multi-lineage progenitors to mimic the normal composition of cell types during brain development. The system has successfully predicted known toxins in blind screens. Due to the technical difficulty in establishing and maintaining organoid cultures, 2D cell models still dominate the early phases of drug development, such as the lead generation phase. However, due to their enhanced ability to mimic human physiology, organoid cultures are especially valuable in lead optimization and preclinical development.

Increasing demands from patient advocacy groups and the anticipation of changing government policies to introduce new competition among pharmaceutical companies have continuously added tremendous pressure to reduce drug prices and a demand to enhance cost effectiveness in the drug development pipeline. Stem cell differentiation relies heavily on growth factors, which are expensive and short-lived. 2D differentiation allows easy access of growth factors to cultured cells and, in many cases, allowed close to homogeneous induction of progenitor cell types from human ESCs and iPSCs. Coupling an initial 2D differentiation protocol followed by organoid formation would enhance cost-effectiveness. On the other hand, small molecules have been developed to replace growth factors. They are smaller in molecular mass, cheaper and more stable than growth factors. A study testing small molecules for replacing growth factors used in hepatic differentiation of hESCs has shown that the cost could be reduced by 67% (Tasnim et al., 2015). Small molecules will have better ability to penetrate organoid structures compared to growth factors. Therefore, they are better suited to be employed at later stages of organoid cultures.

5.3 FACILITATING THE SETUP OF VARIOUS BIOENGINEERING APPROACHES. In static cultures, cells in organoids do not experience mechanical signals as

they do in the body. Dynamic (perfusion) cultures can provide the necessary fluid shear stress and other forces. They also can facilitate the efficient delivery of nutrients and oxygen. Perfusion bioreactors, which allow cultures in much larger scales than microfluidic devices, have been used in tissue engineering for bones (Gaspar et al., 2012) and intestinal tissues (Kim et al., 2007). A recent study reports human liver organoids formed with diameters up to a few millimeters from the self-organization of a conglomerate of genetically engineered adult hepatocytes, liver sinusoidal endothelial cells, and mesenchymal stem cells in a perfusion bioreactor (Ramachandran et al., 2015), suggesting that the principle of self-organization might be applicable to adult cell populations. On the other hand, microfluidic devices have also been used for perfusion cultures. Organ-on-a-chip is a microfluidic cell culture device with hollow microchannels onto which cells are cultured and through which medium is flowed (Bhatia and Ingber, 2014). Organ-on-a-chip devices can be used to culture one or more types of cells simultaneously and to mimic pathophysiological conditions at different levels, such as at the tissue/organ level, where cells from the same tissues and organs are cultured on a chip, or at organism level, where cells from different tissues and organs are used (Huh et al., 2011). Organ-on-a-chip and human-on-a-chip approaches enable high throughput assays. By using an organ-on-a-chip device, the organoid-based *in vitro* model can predict drug response and toxicity at an organismal level. A pioneering human-on-a-chip study has combined multiple cell types in a multi-channel 3D microfluidic cell culture system (Zhang et al., 2009). This system included cell lines representing liver, kidney, lung, and adipose tissues grown in separate channels and cultured with a common medium mimicking blood flow. Similarly, different types of organoids could be cultured in such a multi-channel microfluidic device to build a human-on-a-chip. Organoids could be formed in other culture systems and then transferred to the microfluidic device or could be formed directly in the microfluidic device. Two recent reviews have described the potential of organoid-on-a-chip in biomedical research and applications (Konar et al., 2016; Skardal et al., 2016). Microfabrication can produce topological patterns that are particularly useful for the study of neuronal behavior. Jeong, et al. have created a deep hemicylindrical, microchannel-networked, concave array system for the formation of nerve-like networks (Jeong et al., 2015). Rat embryonic neural progenitor cells self-aggregated into host neurospheroids in concave microwells and satellite neurospheroids in deep hemicylindrical channels. Neurites grew along channels and were bridged by satellite neurospheroids to connect host neurospheroids together. The neural network was shown to transmit signals from one neurospheroid to another. Taken together, other components of our approach, such as the use of empirically tested design matrices to control the microenvironments and the administration of maturation factors specific for different organs and tissues, should also be considered in these systems.

5.4 MEETING THE DEMANDS FOR HUMAN TISSUES FOR TRANSPLANTATION. Demand for raw tissue materials for transplantation calls for a supply of *in vitro* derived, xeno-free, and high-quality human tissues. For instance, for end-stage organ failure, such as liver and heart failure, organ transplantation is the only available treatment. Renal replacement therapy can treat end-stage renal diseases, but kidney transplantation represents the best treatment both for patients' quality of life and cost-effectiveness. Over 4,500 people in Canada (2015 data, https://www.cihi.ca/en/types-of-care/specialized-services/organ-replacements/e-statistics-on-organ-transplants-waiting), 120,000 people in the U.S. (2015 data, www.organdonor.gov), 86,000 in the European Unions plus Iceland, Norway, and Turkey (December 2013 data, http://ec.europa.eu/health//sites/health/files/blood_tissues_organs/docs/ev_20141126_factsfigure

s_en.pdf), and 1.5 million in China (2007 data, http://www.ghgj.org/Living%20Organ%20Transpl.pdf) are on the waiting list for organ transplant. Most of these people need kidney transplantation (>80%), followed by liver (~10%), heart (<5%), lung (~1%), and pancreas (~1%). In the U.S., from early 1990s onwards, the gap between the number of people waiting for a transplant and the number of organ donors has continued to widen to over 7 times. Added to the demand, organ donation is still less common in many countries such as China and Japan. Apart from internal organs, there is also increasing demand for human cell and tissue materials to regrow missing bones, muscles, connective tissues, neural plexi, and skin in the face, neck, and extremities resulting from injuries in wars, motor vehicle-related accidents, burns, and natural disasters (Jalali et al., 2014; van Zuijlen et al., 2015).

There are ongoing clinical trials sponsored by the U.S. NIH to conduct transplantation trials using human ESC- and iPSC-derived cardiac progenitors and retinal pigmented epithelial cells (www.clinicaltrials.gov). Clinical data for other more highly demanded tissues for transplantation such as kidney, liver, and pancreas are urgently needed. In animal studies, xenographic transplantation into adult mouse tissues of human liver organoids generated from a composite aggregate of liver progenitors, endothelial cells, and mesenchyme stem cells has been conducted with success (Takebe et al., 2014). To extend its application, such a multi-lineage recombination method using organ-specific progenitors and vascular and mesenchymal stem cell types has been used to successfully reconstruct composite organoids for intestine, lung, kidney, heart, and brain (Takebe et al., 2015). Liver and other organoids derived from these and other studies suffer from a limitation in size, which usually ranged in the millimeter scale, thereby restricting their direct usage in transplantation. To resolve this issue, de-cellularized liver scaffolds from animals and human cadavers or cell-free scaffolds generated from 3D printing technology could be made and seeded with dissociated organ-specific precursors or directly with organoids containing parenchymal, vascular, and other supporting cell types (Collin de l'Hortet et al., 2016). Together with physiologically relevant culturing methods and bioreactor cultures, these scaffolds could physically support the growth of larger organ structures *in vitro*. Of interest, whole organ de-cellularization has been achieved for liver and a number of other organs including heart, lung, kidney, and pancreas (Scarritt et al., 2015). One of the main challenges in this type of study is to maintain the architecture and composition of the ECM during the de-cellularization process, such as collagen, laminin, elastin, and fibronectin, and biologically-active molecules, such as growth factors. Synthesizing cell-free scaffolds with xeno-free designer matrices and 3D printing technology is an alternative to using de-cellularized organ scaffolds. A few studies have attempted to re-cellularize lung and kidney scaffolds derived from rhesus monkey's organs with undifferentiated human ESCs (Scarritt et al., 2015). The resultant chimeric organs contain ESCs expressing non-specific markers. Differentiated ESC- and iPSC-derived organ progenitors can instead be used for re-cellularization to promote tissue-specific differentiation. For advanced clinical studies, patient-specific iPSCs derived with xeno-free methods should be used to respond to ethical concerns regarding preservation of human embryos and to reduce chances of immune rejection upon transplantation.

6 CONCLUSION

In this review, we have highlighted new perspectives and directions that have been initiated in recent organoid studies. The new development will act as a catalyst for the organoid field and provide new directions to other fields, particularly for developmental biology, embryonic stem cell studies, and adult stem cell biology.

By applying the principle of reverse engineering, we propose a new pipeline for human organoid generation that potentially can greatly enhance the efficiency of generation, the physiological relevance, and the functionality of *in vitro* derived human organoids. By exploiting the self-organizing property of embryonic progenitors, the dissociation-aggregation approach allows the generation of organoids with enhanced native cellular composition as well as more flexible experimental designs and multi-stage quality controls as progenitor raw materials can be generated in batches, cryopreserved, thawed, and expanded before organoid formation. Designer matrix, whether it is tailor-made, native or a composite of both, helps construct the native micro- and matrix environment organoid cell types encountered during aggregation, self-organization, and differentiation. Lastly, a rigorous maturation schematic for different organoid systems permits the generation of fully-functional and terminally-differentiated cell types from human organoids that can be used in a wider range of applications and more closely mimic normal human physiology.

To significantly advance human organoid synthesis technology for various applications, there is an urgency to synthesize knowledge and technologies developed in different fields including stem cell biology, developmental biology, matrix biology, systems biology, bioengineering, material science, biostatistics, and bioinformatics. Such endeavors have been made in various fronts and have to be continuously encouraged by government institutions, sponsoring institutions, and other private funding agencies. Continuous education of the public and communications among scientists, the public media, and the public are also paramount in reducing unwarranted skepticism for using stem cell-derived cell types and tissues for basic research and biomedical applications. Various scientific fields, when proposing research, on the other hand, should also avoid over-optimism and fairly evaluate the beneficial effects of stem cell products.

ACKNOWLEDGEMENT

We would like to express our sincere appreciation to Cayla O. Broton, Raman Nelakanti, Dr. Andrzej Ciechanowicz, and Dr. Marissa Holmbeck for critical reading of our manuscript and to Dr. Patrick Tam (Children Medical Research Institute, Australia) for providing valuable comments and criticism. A.W.L. is grateful to Drs. Martin Garcia-Castro and Andrew Xiao for their continuous support of his research. A.W.L. is supported by funding from Connecticut Innovations and the National Institute of Health. The sponsor plays no role in the study design, collection, analysis, and interpretation of data, neither has it involved in the writing of the report and in decision to submit the article for publication.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure Captions

Figure 1. A novel pipeline for developing future protocols to generate organoids.

Figure 2. Increased control on the timing of progenitor incorporation into organoid structures.

Figure 3. Dissection of the self-organization process.

1708 **Table 1 – Summary of pioneered animal studies for organ progenitor aggregation**

Germ layer	Tissues	Species	Embryonic stage	Length of culture (days)	Reference
Ectoderm	Inner ear	Gallus gallus			(Orr, 1968)
	Inner ear	Mus musculus	E13-E14	6	(Bianchi et al., 2002)
	Cerebral cortex, brainstem spinal cord	Mus musculus			(Crain and Bornstein, 1972)
	Retina	Gallus gallus			(Layer et al., 2001)
	Submandibular gland	Mus musculus	E13-E17	4	(Wei et al., 2007)
Mesoderm	Kidney	Mus musculus	E11.5	21	(Benedetti et al., 2016; Xinaris et al., 2012)
	Heart	Gallus gallus	2.5, 4 or 7d		(McDonald and Sachs, 1975)
		Gallus gallus, Rat	50h, 12d		(Nag et al., 1980)
Endoderm	Pancreas	Mus musculus	E10.5	14	(Greggio et al., 2013; Greggio et al., 2014)

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1710 **Table 2. Adult stem cell-derived organoids**

Germ layer	Tissue	Species	Niche factors for expansion	Matrix	Conditions for *in vitro* differentiation	Reference
Endoderm	Intestine	Mouse	EGF, Noggin, R-spondin 1, Y-27632 (for single cell culture)	Matrigel	Same as expansion condition	(Sato et al., 2009)
Endoderm	Intestine	Human	EGF, Noggin, R-spondin 1, Wnt3A, gastrin, nicotinamide, A-83-01, SB202190	Matrigel	Withdrawal of Wnt3A, nicotinamide and SB202190	(Sato et al., 2011)
Endoderm	Colon	Mouse	EGF, Noggin, R-spondin 1, Wnt3A, Y-27632 (for single cell culture in the first 2 days)	Matrigel	Withdrawal of Wnt3A	(Sato et al., 2011)
Endoderm	Colon	Human	EGF, Noggin, R-spondin 1, Wnt3A, gastrin, nicotinamide, A-83-01, SB202190	Matrigel	Withdrawal of Wnt3A, nicotinamide and SB202190	(Sato et al., 2011)
Endoderm	Stomach	Mouse	EGF, Noggin, R-spondin 1, Wnt3A, gastrin, FGF10, Y-27632 (for single cell culture in the first 2 days),	Matrigel	Wnt3A reduction	(Barker et al., 2010)
Endoderm	Stomach	Human	EGF, Noggin conditioned medium, R-spondin 1 conditioned medium, Wnt conditioned medium, gastrin, FGF10, A-83-01, nicotinamide (for single cell culture), Y-27632 (for single cell culture)	Matrigel	Nicotinamide for gland-type organoids; Withdrawal of Wnt for pit-type organoids	(Bartfeld et al., 2015)
Endoderm	Liver	Mouse	Gastrin, EGF, R-spondin 1, FGF10, nicotinamide, HGF, Y-27632 (for single cell culture in the first 4 days), Noggin and Wnt3a (for the first 4 days)	Matrigel	Withdrawal of R-spondin 1, HGF and nicotinamide and addition of EGF, FGF10, A-83-01, DATP and dexamethasone	(Huch et al., 2013b)
Endoderm	Liver	Human	Gastrin, EGF, R-spondin 1 conditioned medium, FGF10, nicotinamide, HGF, A-83-01, Forskolin (a cAMP pathway agonist); For the first 3 days also including Y-27632, Noggin and Wnt conditioned medium	Matrigel	First BMP7 for 2-4 days and then the withdrawal of N-acetylcysteine, R-spondin 1, FGF10, nicotinamide and Forskolin and addition of FGF19, DAPT and dexamethasone	(Huch et al., 2015)
Endoderm	Pancreas	Mouse	Gastrin, EGF, R-spondin 1, Noggin, FGF10, nicotinamide, Y-27632 (for single cell culture in the first 4 days)	Matrigel	In suspension 0.2% FBS, Activin A for 3 days, then with indolactam-V, FGF10, 2% FBS for 4-5 days, and then with B27, Noggin, retinoic	(Huch et al., 2013a)

					acid, KAAD-cyclopamine for 6 days, finally with B27 and DBZ for 2-4 days before *in vivo* transplantation in kidney capsule	
Endoderm	Pancreas	Mouse	Gastrin, EGF, R-spondin 1, Noggin, FGF10, nicotinamide, Y-27632 (for single cell culture in the first 4 days)	Matrigel		(Boj et al., 2015)
Endoderm	Pancreas	Human	EGF, R-spondin 1 conditioned medium, Wnt3a conditioned medium, Noggin conditioned medium, gastrin, FGF10, nicotinamide, A-83-01, prostaglandin E2	Matrigel		(Boj et al., 2015)
Endoderm	Lung	Human	10% FCS, hydrocortisone, insulin, transferrin, triiodothyronine, choleragen, adenine, EGF	3T3-J2 feeder	Self-assembly or Matrigel with retinoic acid, cholera toxin, knockout serum replacement	(Kumar et al., 2011)
Endoderm	Lung	Mouse	Insulin, transferrin, cholera toxin, EGF, bovine pituitary extract, 5% FBS, retinoic acid	Matrigel at air-liquid interface	Coculture with primary PDGFRα$^+$ lung stromal cells	(Barkauskas et al., 2013)
Endoderm	Prostate	Mouse/human	EGF, Y-27632, 5% Matrigel, 5% charcoal-stripped FBS, DHT	Flotation	Same as expansion condition	(Chua et al., 2014)
Endoderm	Prostate	Mouse	EGF, Noggin, R-spondin, A-83-01, DHT	Matrigel	Same as expansion condition	(Karthaus et al., 2014)
Endoderm	Prostate	Human	EGF, Noggin, R-spondin, A-83-01, DHT, FGF10, FGF2, prostaglandin E2, nicotinamide, SB202190	Matrigel	Same as expansion condition	(Karthaus et al., 2014)
Ectoderm	Mammary gland	Mouse	Insulin, hydrocortisone, prolactin, 1% FCS	Matrigel	Same as expansion condition	(Shackleton et al., 2006)
Ectoderm	Mammary gland	Mouse	Hydrocortisone, insulin, EGF, FGF2, FGF10, Wnt3A, heparin, R-spondin 2, Y-27632	Basement membrane extract	Same as expansion condition	(Jamieson et al., 2016)
Ectoderm	Retina	Mouse	With or without FGF2	Suspension	On poly-L-ornithine-coated glass in EGF containing serum-free medium	(Tropepe et al., 2000)
Ectoderm	Salivary gland	Mouse	EGF, FGF2, insulin, dexamethasone, Y-27632	Matrigel	Intact spheres in collagen-Matrigel mixture (4:6) and	(Nanduri et al., 2014)

Mesoderm	Fallopian tube	Human	Wnt3A conditioned medium, R-spondin 1 conditioned medium, EGF, noggin, FGF10, nicotinamide, Y-27632, SB431542	Matrigel	10% FCS containing medium Same as expansion condition	(Kessler et al., 2015)

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